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The Effects of Repeated Low-Dose Sarin Exposure

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ABSTRACT

This project assessed the effects of repeated low-dose exposure of guinea pigs to the organophosphorus nerve agent sarin. Animals were injected once a day, 5 days per week (Monday-Friday), for 2 weeks with fractions (0.3 x, 0.4 x, 0.5 x or 0.6 x) of the established LD_{50} dose of sarin (42 μg/kg, s.c.). The animals were assessed for changes in body weight, red blood cell (RBC) acetylcholinesterase (AChE) levels, neurobehavioral reactions to a functional observational battery (FOB), cortical electroencephalographic (EEG) power spectrum, and intrinsic acetylcholine (ACh) neurotransmitter (NT) regulation over the 2 weeks of sarin exposure and for up to 12 days post-injection. No guinea pig receiving 0.3, 0.4 or 0.5 x LD_{50} of sarin showed signs of cortical EEG seizures despite decreases in RBC AChE levels to as low as 10% of baseline, while seizures were evident in animals receiving 0.6 x LD_{50} of sarin as early as the second day; subsequent injections led to incapacitation and death. Animals receiving 0.5 x LD_{50} sarin showed obvious signs of cholinergic toxicity; overall 2 of 13 animals receiving 0.5 x LD_{50} sarin died before all 10 injections were given, and there was a significant increase in the angle of gait in the animals that lived. By the 10^{th} day of injection, the animals receiving saline were significantly easier to remove from their cages and handle and significantly less responsive to an approaching pencil and touch on the rump in comparison with the first day of testing. In contrast, the animals receiving 0.4 x LD_{50} sarin failed to show any significant reductions in their responses to an approaching pencil and a touch on the rump as compared with the first day. The 0.5 x LD_{50} sarin animals also failed to show any significant changes to the approach and touch responses and did not adjust to handling or removal from the cage from the first day of injections to the last day of handling. Thus, the guinea pigs receiving the 0.4 and 0.5 x LD_{50} doses of sarin failed to habituate to some aspects of neurobehavioral testing. Spectral analysis of EEG data suggested that repeated sarin exposure may disrupt normal sleeping patterns (i.e., lower frequency bandwidths). While these disruptions returned to relative normalcy 6 days after the last injection in animals receiving 0.4 x LD_{50} sarin, a significant deficit was still observed in the animals that received 0.5 x LD_{50} sarin. Ten to twelve days after the last sarin injection (in 0.4 x LD_{50} group only), neurochemical data showed that striatal choline levels were reduced in comparison to the saline group. At this time atropine sulfate (5 mg/kg, i.p.) challenge resulted in a transient elevation in striatal ACh levels in animals exposed to repeated 0.4 x LD_{50} sarin as well as in control animals. No evidence of brain or heart pathology was found in any guinea pig that survived all ten sarin injections.

INTRODUCTION

Chemical warfare nerve agents (CWNAs), such as sarin, soman and VX, are organophosphorus compounds (OPs). They disrupt normal nervous system transmissions through the irreversible inhibition of acetylcholinesterase (AChE), the enzyme that breaks down the cholinergic neurotransmitter (NT) acetylcholine (ACh). The buildup of ACh in response to a large exposure to nerve agents can lead, unless promptly treated, to muscle weakness, increased secretions (i.e., lacrimation, rhinorrhea, salivation), convulsions and seizures, respiratory depression, coma and death (Taylor, 1996). The progression of signs, their neuropharmacological basis, and toxic consequence elicited from acute high-dose exposures has been well characterized (McDonough and Shih, 1993, 1997; Shih et al., 2003). However, much less is known about the long-term effects of repeated low-dose CWNA exposure. Comprehensive reviews of the available literature on the long-term health effects of exposure to low level CWNAs have been published (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, 1985; Moore, 1998; Romano et al., 2001).

There have been previous animal studies investigating the neurobehavioral effects of long-term low-level exposure to OP AChE inhibitors (see Jamal et al., 2002 for a review). For example, Prendergast et al. (1998) demonstrated that long-term OP exposure (>5 days) leads to memory deficits in the rat. These animals were exposed to diisopropylfluorophosphate (DFP) (0.25 mg/kg/day, s.c.) for 14 days. The DFP-treated rats showed significant declines, as compared with controls, in their abilities to initially learn a spatial recognition task. In the great majority of the available literature on repeated low-dose exposure to CWNAs, soman is the OP studied most often. Repeated low-dose soman exposure has been investigated in mice (Sterri et al., 1981), rats (Sterri et al., 1980; Dulaney et al., 1985; Hymowitz et al., 1985; Kerenyi et al., 1990; Shih et al., 1990; Howerton et al., 1991), guinea pigs (Sterri et al., 1981, 1982), and primates (Gause et al., 1985; Blick et al., 1991, 1994). The effects of the repeated soman exposures, cited above, ranged from performance decrements on a well-learned compensatory tracking task (Blick et al., 1994) to development of attention deficits (Gause et al., 1985) to hyper-reactive responses to handling (Shih et al., 1990).

Unlike soman, the nerve agent sarin has been used previously by extreme terrorist groups (e.g., 1995 Tokyo subway incidence (Nozaki et al., 1995)) and on the battlefield (Macilwain, 1993; Brown and Brix, 1998). More than 98,000 Persian Gulf War veterans have been notified that they were potentially exposed to a plume of sarin when American forces destroyed an ammunition depot shortly after the end of the 1991 Gulf War (Enserink, 2001). Additionally, there is a worldwide movement toward destruction of chemical weapons stockpiles that are maintained by more countries than ever before (Marrs et al., 1996), and the possible long-term neurobehavioral effects of trace nerve agent exposure on the workers is virtually unknown. In general, exposure to low-level CWNAs is a potential emerging health hazard that requires further investigation (Romano et al., 2001).

Despite these facts, the amount of literature regarding the effects of repeated low-level exposure to sarin is rather sparse. Burchfiel and his colleagues (1976) exposed rhesus monkeys to repeated low levels of sarin (1 μg/kg, i.m.) once per week for 10 weeks. Despite increases in high frequency beta activity upon electroencephalographic (EEG) analysis, there were no signs of adverse health or long-term behavioral effects. In contrast, when sarin was administered subcutaneously (s.c.) to rats once per day for up to 85 days, doses less than 0.3 x LD_{50} resulted in significant reductions in body weight gains by as early as the 7^{th} day of injections (Dulaney et al.,

1985). In the same study, a dose of approximately 0.36 x LD_{50} resulted in the death of 4 out of 11 animals by the 10^{th} day of injections. Husain et al. (1993), using a repeated inhalation protocol (5 mg/min/m^3 for 20 minutes per day for 10 days) in mice, showed that sarin exposure amounting to less than 0.2 x LC_{50}/day resulted in delayed (14^{th} day after exposure) muscle twitching, weakness in the extremities and slight ataxia. It has been observed in rats that intraperitoneal (i.p.) injections of subtoxic doses of sarin or soman decreased locomotor activity, altered behavior on the plus-maze and elevated horizontal bridge tests (Sirkka et al., 1990; Nieminen et al., 1990). Similar results of decreased locomotor activity and altered behavior on the plus-maze were reported after low to mild doses of soman exposure in mice (Baille et al., 2001). In a series of studies on the effects of repeated low-level sarin inhalation in rats, Kassa et al. (2001a, b) concluded that clinically asymptomatic doses were disruptive to neurophysiological function and caused long-term memory impairments. Two related studies reported long-term alterations (up to 12 months) in immune function and liver nucleic acid metabolism (Kassa et al., 2000, 2001c), whereas Conn et al. (2002) reported that one hour per day low-level sarin inhalation exposure did not change body temperature and locomotor activity during exposure or for one month post-exposure.

It was hypothesized as early as 1969 (Metcalf and Holmes, 1969) that long-term exposure to OPs can induce irreversible or slowly reversible brain dysfunction. Indeed, this theory has been supported by studies investigating EEG changes in response to long-term OP exposures in both non-human primates (Burchfiel et al., 1976; Duffy, 1980; Burchfiel and Duffy, 1982) and humans (Metcalf and Holmes, 1969; Duffy et al., 1979; Wadia et al., 1974; Duffy, 1980; Burchfiel and Duffy; 1982).

Changes in both extracellular NT levels measured by *in vivo* microdialysis techniques and total NT levels measured in brain homogenates, following exposure to seizure-inducing doses of CWNAs, have been documented in animal studies. Upon exposure to CWNAs, increases in total brain ACh levels are immediate (Shih, 1982; Fosbraey et al., 1990, 1991; Shih et al., 1993; Shih and McDonough, 1997), but return to baseline levels extracellularly within 90 minutes of seizure onset (Lallement et al., 1992). An increase in dopamine metabolites, i.e., 3,4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA), is evident approximately 10 minutes after seizure onset with increases in HVA continuing for up to 80 minutes after seizure onset (Shih and McDonough, 1997; McDonough and Shih, 1997). Increases in extracellular glutamate have also been reported in numerous brain regions following soman-induced seizure onset (Lallement et al., 1991). These data, taken together, support the idea that there is a triphasic NT model for onset and progression of seizures and subsequent brain damage upon acute exposure to OP ChE inhibitors (McDonough and Shih, 1997). The initial phase is modulated by cholinergic stimulation and begins with exposure to the OP lasting until approximately 5 minutes after seizure onset. From 5 minutes to 40 minutes there is a transition phase consisting of both cholinergic and non-cholinergic modulation. After 40 minutes the seizure maintenance is believed to be primarily non-cholinergic. Whether there are changes in brain NT function and levels upon repeated low-dose exposure to CWNA is much less clear. Most studies of long-term assessment of NT levels have been performed in a paradigm where there is only a single sub-seizure threshold exposure to these agents. Furthermore, there have been no measurements of extracellular NT levels (cholinergic or non-cholinergic) after day 5 of continued low-level OP exposure. Although it has already been suggested that chronic exposure to soman may result in down-regulation of cholinergic receptors (Modrow and McDonough, 1986), the effects that chronic elevation of extracellular NTs, such as ACh and glutamate, might have on receptor

numbers or on responses to agonists have yet to be defined. Additionally, the majority of the reports based on animal studies support the concept that if seizures are not induced there is no resultant brain damage and that low sub-seizure doses of a single nerve agent do not elicit neuropathology (McDonough et al., 1995).

Ongoing medical research and development of antidotes against the toxic effects of CWNAs in our laboratory has been focused on the guinea pig animal model (Atchison et al., 2004; Shih et al., 2003). Guinea pigs are considered a more valid rodent model for studying the toxicological effects of nerve agents and for predicting the efficacy of treatment for CWNA poisoning in primate species than are mice or rats (Inns and Leadbeater, 1983). Rats and mice possess large amounts of carboxylesterase enzyme, which nonspecifically binds nerve agents, so they require substantially higher acute doses of these agents to produce equivalent toxic effects than do guinea pigs or higher species such as nonhuman primates (Maxwell et al., 1987).

One of the objectives of the current study was to develop a model for repeated sarin exposure in the guinea pig that did not elicit severe signs of intoxication (e.g., tremors, epileptiform seizures, or death) when injected once per day over a 2-week (Monday - Friday) period. This model was then used to assess whether subtle neurobehavioral and/or physical deficits develop and persist in the animals exposed to the 2 weeks of sarin injections. As a positive control, a dose group that did develop overt signs of sarin intoxication was also included in this report.

Additionally, we hypothesized that the long-term abnormalities associated with repeated exposure to low-dose OPs, previously identified in EEG spectra (Burchfiel et al., 1976), are most likely linked with alterations in normal neuropharmacological homeostasis. Therefore, these studies have been further designed to expand this model of repeated non-toxic sarin exposure in guinea pigs to investigate what effect our dosing regimen has on EEG power spectra and to determine whether the animals exhibit predictable neurochemical changes in response to drugs that result in changes in the intrinsic NT regulation.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (Crl:(HA)BR; 290-430 g starting weight), obtained from Charles River Labs (Kingston, NY), were used for these studies. Upon arrival, the animals were quarantined for a week and tested for evidence of disease. They were individually housed in polycarbonate cages in temperature (21 ± 2 ^{o}C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12-h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available whenever the animals were in home cages. The research environment and protocols for animal experimentation were approved by the institutional animal care and use committee. The animal care program at this institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Surgery

Guinea pigs were anesthetized with isoflurane and surgically implanted with cortical screw electrodes using standard small animal aseptic surgical techniques reported previously (Shih and McDonough, 1999). Small burr holes were drilled in the skull to accept stainless steel screw electrodes (equidistance between bregma and lamda and 3 mm lateral to the midline suture) and

an intracerebral guide cannula (BAS #MD-2250) for a microdialysis probe. The tip of the guide cannula was lowered into the coordinates (AP = +11.4; L = +3.6; Depth = -4.6; Level = 6.0) based on Paxinos and Watson (1986) for caudate nucleus. The electrode leads were connected to a miniature connector plug. The wire, screws, plug, and guide cannula were held in place using cranioplastic cement. The guinea pigs were allowed to recover for 10-14 days before experiments began.

Materials

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs, Inc. (Berkeley, CA). Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). It was diluted in saline in concentrations to deliver injection volumes = 0.5 ml/kg, and the diluted solution was maintained on ice prior to administration. The institute's historic database indicated that the LD_{50} for sarin in the guinea pig is in the range of 38 - 46 µg/kg (s.c.). An initial experiment was conducted to verify the LD_{50} dose of sarin in guinea pigs, which was determined to be 42 µg/kg (s.c.). Subsequent dosing was based on this value. Atropine sulfate and pentobarbital sodium were purchased from Sigma-Aldrich Co. (St. Louis, MO) and were diluted in saline before administration. Injection volume was 0.5 ml/kg for saline and atropine sulfate. Isoflurane (Attane®, USP) was obtained from Minrad, Inc. (Bethlehem, PA). O-phosphoric acid (85%, HPLC grade), disodium ethylenediamine tetraacetate (EDTA), and 10% neutral buffered formalin were purchased from Fisher Scientific (Pittsburgh, PA). Neostigmine bromide, acetylcholine chloride, and choline chloride were purchased from Sigma-Aldrich Co. All other chemicals used were analytical reagent grades.

Experimental Protocol

Guinea pigs received (s.c.) selected doses of sarin or saline (as control) once per day, 5 days per week (Monday - Friday) for 2 weeks. The injections were administered under the skin of the back in a volume of 0.5 ml/kg body weight. The s.c. route of administration was chosen based on its extensive use by other investigators on the effects of chronic nerve agent exposure and the fact that there is minimal first-pass detoxification of the agent by the liver by this route (Dulaney et al., 1985; Shih et al., 1990; Sterri et al., 1980, 1981, 1982). A pilot study was first performed to determine what dose of sarin could be repeatedly injected over the 2-week period. A predetermined criterion for the experiment was to determine the maximum tolerated dose (MTD) of sarin that did not elicit signs of nerve agent intoxication. The doses of sarin tested in this pilot study were 0.3, 0.4, 0.5 and 0.6 x LD_{50} (n = 4 animals at each dose). Baseline EEG recordings were taken for 10 minutes prior to all sarin injections, and EEG recordings were continued for an additional 40 minutes following sarin injections. In the pilot study the guinea pigs were monitored for epileptiform EEG activity, changes in body weight, and red blood cell (RBC) AChE levels. From the results obtained in the pilot study the 0.4 x LD_{50} and 0.5 x LD_{50} doses of sarin were chosen as the doses to be injected in the full experiment, along with saline for the controls. In the full study, guinea pigs were randomly assigned to treatment groups (saline, 0.4 x LD_{50} or 0.5 x LD_{50} sarin). The experiment was run in a series of replications, with each replication consisting of 8-10 guinea pigs randomly distributed between the 3 treatment conditions. In the full experiment, the same regimen of sarin dosing and monitoring of EEG, body weight and RBC AChE was used. In addition, the animals were evaluated with a functional observational battery (FOB) to determine neurobehavioral functions such as righting response, motor, sensory and physical deficits. FOB evaluation was performed on the animals

prior to the 1st sarin or saline injection (baseline) and then after the sarin or saline injections on the 3rd, 5th, 6th, 8th and 10th days. It was also performed on the 4th and 6th days following termination (recovery period) of injections. FOB was performed immediately after the EEG evaluation, 40 - 50 minutes after injections on exposure days. Blood was drawn for RBC AChE determinations prior to the 1st and 6th (following weekend recovery) sarin or saline injections and 2-3 hours after the 2nd, 5th, 7th and 10th injections. The animals were allowed to recover for 21 days after the last injection with RBC AChE measurements taken on the 4th, 7th, 14th and 21st days of recovery. To minimize animal discomfort the blood draws in the guinea pigs were staggered such that blood was not drawn from every animal on every day of blood collection.

AChE Assay

RBC AChE activity was assessed by collecting approximately 0.5 ml of blood via toe-nail clip (Vallejo-Freire, 1951). Whole blood was prevented from clotting by the addition of a small amount (15 µl) of EDTA (4 g/L). Whole blood was separated into plasma and RBC by centrifugation (11 minutes, 14000 x g). RBC AChE activity was determined, using acetylthiocholine iodide as a substrate, by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman et al. (1961) and modified for the COBAS/FARA by Hobson et al. (1988).

Neurobehavioral (FOB) Testing

The FOB is a sequence of rapid tests (completed in 6-8 minutes) used to assess neurological functions. It allows for the qualitative and quantitative evaluation of the behavioral and physiological effects of neurotoxicants (Bowen and Balster, 1997; Moser et al., 1988; Tegeris and Balster, 1994; Youssef and Santi, 1997). Two technicians, who were unaware of the treatment of the animals, performed all FOB scoring. The order of animal selection for the neurobehavioral testing was performed randomly by the scorer. The scorers tended to test the animals in numerical order, from lowest to highest, based on their identification numbers. The scoring sheet (see Appendix) was adapted from those previously published (Moser et al., 1988; Youssef and Santi, 1997), with slight modifications for guinea pigs. The specific sequence of testing was as follows.

Home Cage: While in their home cages the guinea pigs were scored positive or negative for the presence of agitation, chewing, tremors, facial dysmorphia and vocalizations. They were graded on a scale for ease of removal, ease of handling and presentation of physical signs, such as fur appearance (piloerection), emaciation, lacrimation and salivation.

Open Field: The animals were placed on top of a lab cart and latency to first movement was timed. The animals were then allowed to move freely for 2 minutes. During this time the animals were scored on their gait description and their level of arousal. The number of grooms, urine spots, fecal matter and rears were counted and recorded.

Reflexes: The guinea pig's responses to an approaching pencil, a tap on the rear and a loud click behind the head were graded. Righting reflex was then measured by placing the animal on its back and recording the time it took for the guinea pig to get to its feet. For the drop reflex, the guinea pig was then dropped, from a supine position, from a height of 30 cm onto a soft landing area. The ease of the landing was scored.

Splay and Gait: The guinea pig's hindlimbs were painted with water-based tempura paint. Hindlimb foot splay was obtained by dropping the guinea pig, from a prone position, from approximately 30 cm high onto a sheet of paper placed on the countertop. The distance between the middle toes of each footpad was measured. The footpads were then repainted and the guinea pigs were placed on a new sheet of paper and allowed to walk freely. The testing was concluded when the guinea pigs maintained forward movement for a minimum of 3 successive steps in a straight path. The angle from the first footstep to the second and third steps was measured.

EEG Recording and Power Spectral Analysis

To record EEG activity, instrumented animals were placed in individual EEG recording chambers (45 cm H x 30 cm W x 25 cm D). Animal headpieces were connected to the EEG recording apparatus. EEG recordings were made using amplifiers and QND software supplied by Neurodata Inc. (Pasadena, CA) (low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz) and displayed on a computer monitor. Baseline EEG recordings were taken for 10 minutes prior to all sarin injections, and EEG recordings were continued for an additional 40 minutes following sarin injections. After sarin injections were terminated (recovery period), EEGs were recorded at various times during recovery. Development of epileptiform seizure activity was operationally defined as the appearance of $\geq$10 seconds of rhythmic high amplitude spikes or sharp wave activity in the EEG.

For power spectral analysis, each recording session of 50 minutes was divided into 7 time periods as follows: BL = the baseline period before injections; IN = 0-6 minutes following injection; P1 = 6-12 minutes following injection; P2 = 12-18 minutes following injection; P3 = 18-24 minutes following injection; P4 = 24-30 minutes following injection; and P5 = 30-36 minutes following injection. Within each time period a contiguous 120-second time snippet was taken for analysis. Each 120-second time snippet was taken as close to the beginning of the time period as possible (with the exception being the BL time period where the snippet was taken in the 120 seconds just prior to the injection being given). The total EEG cortical power consisted of bandwidth powers over 5 different frequency ranges as follows: Delta = 1-3.5 Hz; Theta = 4-7.5 Hz; Alpha = 8-12.5 Hz; Beta I = 13-20.5 Hz; and Beta II = 21-31.5 Hz. Analysis was done on total EEG power as well as on the 5 different frequency bandwidth powers. The means of spectral powers for a particular day, time point and treatment were calculated.

In Vivo Microdialysis

Ten to twelve days following the last sarin or saline injection, a brain microdialysis probe (BR-2 probe, 2 mm membrane, BAS #MD-2200) was inserted into the intracerebral guide cannula, and baseline microdialysis samples were collected (15 minutes per fraction) from the conscious free-moving guinea pigs for a minimum of 1.5 hours, while infusing the caudate nucleus with saline containing 2 µM neostigmine at 3 µl/min. The animals were then injected (i.p.) with 5 mg/kg of atropine sulfate in saline. Microdialysis samples were collected for an additional 2 hours and analyzed for ACh and choline levels.

Determination of ACh and Choline

A BAS200 high-pressure liquid chromatograph (HPLC) with electrochemical detector (Bioanalytical Systems, Inc. (BAS), West Lafayette, IN) was used for determination of ACh and choline in the microdialysates (Huang et al., 1995). Twenty µl of the collected microdialysis perfusates were injected directly into a BAS200 HPLC at a flow rate of 0.05 - 0.1 ml/min in an

isocratic mobile phase that consisted of 14.65 M phosphoric acid (pH 8.5) and 5 ml/l Kathon CG reagent (BAS # CF-2150) in deionized water. An ACh microbore column (1x530 mm ID, 10 µm UniJet, BAS #MF-8904) coupled with AChE/choline oxidase immobilized enzyme reactor (BAS # MF-8903) was used to separate ACh and choline. A "biosensor" was created by coating a glassy carbon electrode with a redox polymer film containing horseradish peroxidase (BAS Peroxidase Electrode Kit, Item No. MF-2095) and operated at +100 mV vs. Ag/AgCl. The redox polymer electrically "wires" the peroxidase to the electrode for the reduction of hydrogen peroxide that was generated from the immobilized enzyme reactor. The detector was set at (-)1.0 and (-)5.0 nanoamps for optimum ACh and choline detection. The cell temperature was set at 35 ^{o}C and the back pressure of this system was at 2000 PSI under optimal conditions. Retention times were approximately 10 min for ACh and 12 min for choline. Samples were quantified using BAS Report Software.

Pathology Evaluation

Within 1 month of the animals' last sarin or saline injection, the guinea pigs were deeply anesthetized with pentobarbital (75 mg/kg, i.p.) and then perfused through the aorta with saline followed by 10% neutral buffered formalin. The fixed brains and hearts were then removed, sectioned and stained with hemotoxylin and eosin (H&E) to assess tissue damage and to verify the location of the tip of microdialysis guide cannula. Brain and heart pathology was analyzed as previously published (McDonough et al., 1995). Animal tissues were evaluated by a board-certified veterinary pathologist who was unaware of the experimental history of a given subject.

Data Analysis

For RBC AChE data and numerical data in the FOB, a one-way analysis of variance (ANOVA) was used to determine whether significant differences existed. For body weight change data, gait angle data and foot splay data a two-way (treatment x day) repeated measures ANOVA was used. Post-hoc Tukey tests were then further used to identify significant effects. For the results of the scored parts of the FOB, Kruskal-Wallis ANOVA on ranks (Hollander and Wolfe, 1973) was used to detect whether there were significant differences between baseline group scores. A Wilcoxon signed-rank test was then used to detect statistical differences between the same individual animals before and after sarin treatment. A difference of $p<0.05$ was considered significant. The EEG power spectra were graphed as a function of day of injections with time period reflected on the z-axis. Each bar reflects the mean for that particular day and time period taken over 120-second time snippet within the time period. A two-factor ANOVA (group: saline vs. sarin exposure; time: 15, 30, 45, 60, 75, 90, 105 and 120 min after atropine dosing), with repeated measures on the time factor, was used for analysis of the ACh microdialysis data.

RESULTS

Pilot Study

None of the lower doses of sarin (0.3, 0.4 and 0.5 x LD_{50}) elicited epileptiform seizure activity during periods of EEG recordings. However, there were noticeable signs (hyper-excitability, muscle tremors, piloerection, chewing) of nerve agent intoxication in all animals receiving the 0.6 x LD_{50} sarin dose (n = 4) by the 2nd day of injections. The 0.6 x LD_{50} sarin dose caused seizures in 2 of the 4 guinea pigs by the 4th day of injections and death in 3 of 4

guinea pigs before the final day of injections. Thus, the 0.6 x LD_{50} dose of sarin did not meet the predetermined criterion and was disqualified for use in further studies. The 0.4 and 0.5 x LD_{50} sarin doses were chosen for full experimentation because they were the highest possible doses that did not elicit cortical EEG seizures nor produce death when given over the 2 weeks of daily injections. The weight gain and RBC AChE data from the 4 animals in each preliminary treatment group were added to the data obtained from the animals in the full experiment. This was done to increase the overall number of animals available for statistical analysis of weight gain and RBC AChE data only. In the full experiment there were 24 animals that received saline or 0.4 x LD_{50} sarin (final total n = 28 for saline and n = 28 for 0.4 x LD_{50}) and 9 animals that received 0.5 x LD_{50} sarin in the full experiment (final total n = 13). The reason for the lower number of guinea pigs receiving 0.5 x LD_{50} sarin is explained below.

Neurotoxicity

While all 4 guinea pigs receiving 0.5 x LD_{50} sarin in the pilot study survived without seizures for the 10 injections, this was not the case in the full experiment, where 2 of the 9 guinea pigs receiving 0.5 x LD_{50} sarin died before the 2 weeks of injections were completed. These 2 animals never showed signs of EEG epileptiform seizures. Therefore, while the 0.5 x LD_{50} sarin dose given in the pilot study met predetermined criteria, the same dose in the full experiment did not. For this reason we terminated further use of the 0.5 x LD_{50} dose of sarin, and the total number of animals given this dose was 13 (4 from the pilot study + 9 from the full experiment).

Weight changes

The guinea pigs showed dose-response changes in body weight over the 2 weeks of injections (Figure 1). The overall average weight gains (calculated as the animal's weight on the 10^{th} day of injections minus the animal's weight prior to the first injection) for the animals receiving saline, 0.4 and 0.5 x LD_{50} sarin were 56.89 ± 2.36, 51.6 ± 2.81 and 30.09 ± 5.25 (mean± SEM) grams, respectively. The two-way ANOVA, with repeated measures on days, showed significant main effects for treatment ($F_{(2, 64)} = 8.18$, $p<0.001$), days ($F_{(8, 512)} = 263.39$, $p<0.001$), and the treatment X days interaction ($F_{(8, 512)} = 8.81$, $p<0.001$). Both the saline control and 0.4 x LD_{50} sarin groups gained significantly greater amounts of weight than did the 0.5 x LD_{50} sarin group throughout the 10-day exposure period. The weight differences first reached significance on day 5, with the 0.5 LD_{50} sarin group weighing significantly less than the saline control group. Over the next week, this growth difference became even more notable, with the 0.5 x LD_{50} sarin group gaining significantly less weight than either the saline controls or the 0.4 x LD_{50} sarin group on injection days 6-10. There was no significant difference between the weight gains of the saline controls and the 0.4 x LD_{50} sarin group on any day during the dosing period.

RBC AChE changes

As shown in Figure 2, by the 2^{nd} day of sarin injections, RBC AChE in the guinea pigs receiving 0.5 x LD_{50} sarin had dropped to 14% of baseline values, which was significantly ($p<0.001$) lower than the 35% of baseline RBC AChE levels in animals receiving 0.4 x LD_{50} sarin. However, by the 10^{th} day of sarin injections RBC AChE levels had dropped to near identical levels (11% vs. 12% of baseline values, respectively) in the 0.4 and 0.5 x LD_{50} animals. The average 2-day weekend (Saturday-Sunday) RBC AChE recovery between the 2 weeks of

sarin injections was 17% and 20%, respectively, for the 0.4 and 0.5 x LD_{50} sarin groups. Even 21 days after termination of the injections the RBC AChE levels of the animals receiving 0.4 and 0.5 x LD_{50} sarin remained at 68% and 67% of control values, respectively, which were still significantly lower than the control AChE levels.

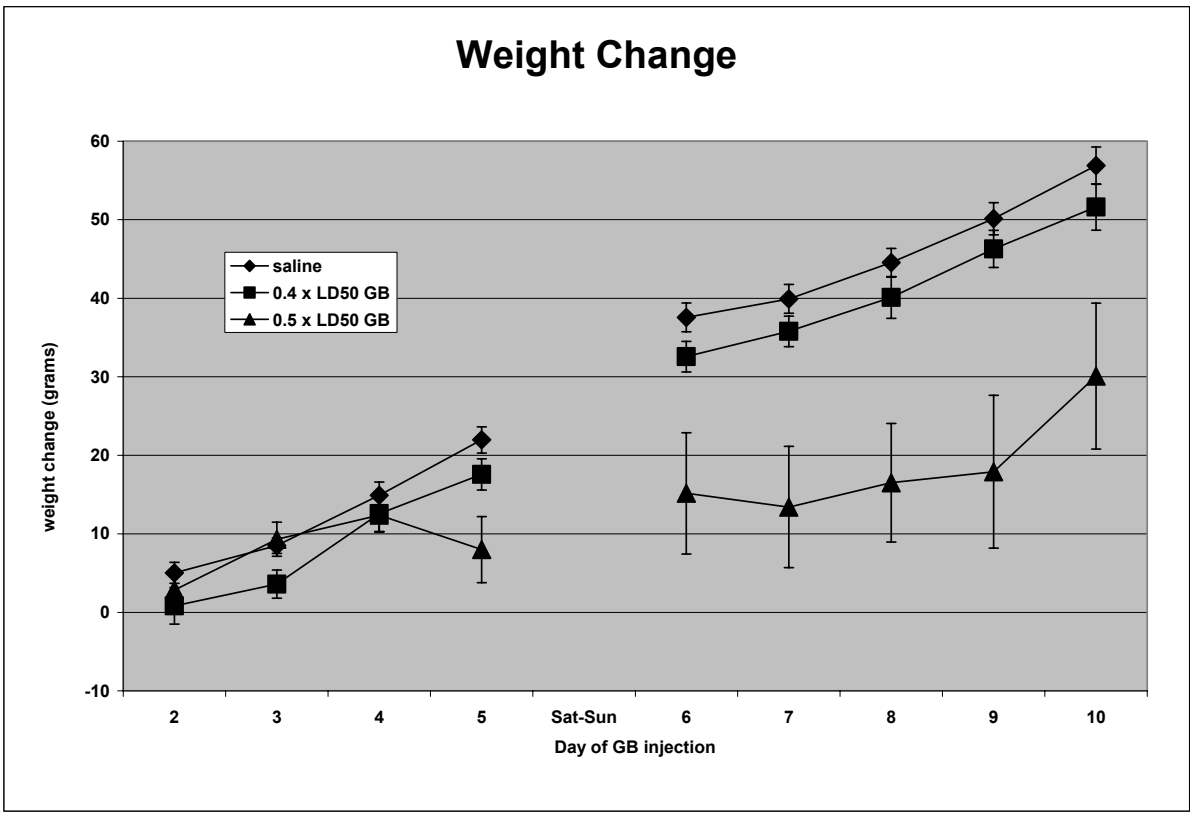


Figure 1. Weight change vs. day of sarin injection. The animals were weighed prior to the first sarin injection and then before each subsequent day's injection. Data are graphed as the change between the initial weight and the weight prior to each day's sarin injections. Values are expressed as means ± S.E.M. N = 28 for saline group, n = 28 for 0.4 x LD_{50} sarin group, n = 11 for 0.5 x LD_{50} sarin group. (There were 13 guinea pigs total, but 2 guinea pigs died before the 8^{th} day of injections and thus were not able to be included in statistical analysis using a 2-way repeated measures ANOVA.) There is a dose-response change in weight in relation to the dose of sarin given. The animals receiving the 0.5 x LD_{50} dose of sarin showed significantly less overall weight gain ($p<0.005$) than the animals receiving either saline or 0.4 x LD_{50} sarin.

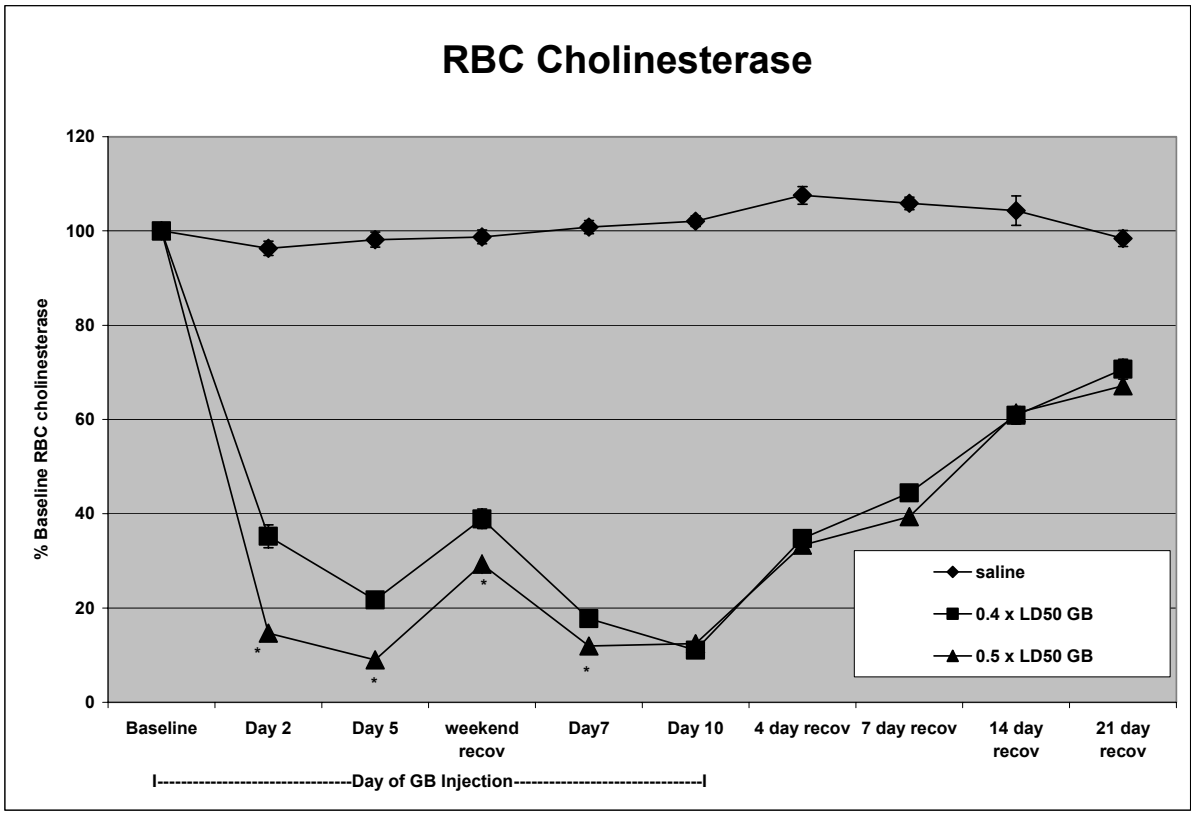


Figure 2. RBC AChE levels vs. day of sarin injection. Blood was taken via toe-nail clip at different times during the sarin injections, and RBC AChE levels were determined by standard methods. To reduce animal discomfort blood was not drawn from every animal on every occasion except for baseline values. Values are expressed as means ± S.E.M. with a minimum n = 7 RBC samples (each run in triplicate) for each data point. By the 2nd day of sarin injections RBC AChE in the animals receiving 0.5 x LD_{50} sarin injections dropped to significantly ($p<0.001$) lower levels than in the 0.4 x LD_{50} sarin animals. However, by the 10th day of sarin injections RBC AChE levels were nearly identical (11% vs. 12% of baseline values) in the 0.4 x LD_{50} and 0.5 x LD_{50} sarin groups. At 21 days recovery RBC AChE levels in the animals receiving 0.4 x LD_{50} and 0.5 x LD_{50} sarin had returned to 68% and 67% of baseline values respectively. * $p<0.01$ vs. 0.4 x LD_{50} sarin group.

Table 1. Summary of the observed changes in Functional Observational Battery scoring after 2 weeks of sarin dosing

	Saline	0.4 x LD50	0.5 x LD50
Cage removal [r]	↓ (easier to remove)*	↓ (easier to remove)*	No sig. change
Handling [r]	↓ (easier to handle)*	↓ (easier to handle)*	No sig. change
Lacrimation [r]	No sig. change	No sig. change	No sig. change
Salivation [r]	No sig. change	No sig. change	No sig. change
Fur appearance [r]	No sig. change	No sig. change	No sig. change
Latency to move [n]	No sig. change	No sig. change	No sig. change
# grooms [n]	No sig. change	No sig. change	No sig. change
# rears [n]	No sig. change	No sig. change	No sig. change
Arousal [r]	No sig. change	No sig. change	No sig. change
Gait [r]	No sig. change	No sig. change	No sig. change
Fecal boluses [n]	No sig. change	No sig. change	No sig. change
Urine spots [n]	No sig. change	No sig. change	No sig. change
Click response [r]	No sig. change	No sig. change	No sig. change
Approach response [r]	↓ (less reactive)*	No sig. change	No sig. change
Touch response [r]	↓ (less reactive)*	No sig. change	No sig. change
Righting reflex [r]	No sig. change	No sig. change	No sig. change
Drop reflex [r]	No sig. change	No sig. change	No sig. change

Table 1. Summary of the observed changes in functional observational battery scoring after the last day of sarin injections as compared with those scores obtained before the first injection (baseline scores). For the ranked data (r) Kruskal-Wallis ANOVA on ranks was used to detect that there were no significant differences between baseline group scores. A Wilcoxon signed-rank test was then used to detect statistical differences between the same individual animals before and after nerve agent treatment. One-way ANOVA was used to analyze numerical data (n) for significance. Guinea pigs that received saline (n=24) became significantly easier to remove from their cages and handle. They also became significantly less reactive to an approaching pencil and to a touch on the rear. In short, they became "habituated" to some aspects of the testing. The average (mean±S.E.M.) baseline scores and scores after the 10^{th} day of injections obtained for each significant score change are referenced within the results section of the text. The guinea pigs receiving 0.4 x LD_{50} sarin (n=24) failed to show any significant decreases in the approach response and touch response. The guinea pigs receiving 0.5 x LD_{50} sarin (n=9 for baseline but 2 animals died before injections were complete) failed to show any significant changes in cage removal, approach response, and touch response and did not adjust to handling. * $P<0.05$.

Neurobehavioral (FOB) Testing

Both the guinea pigs receiving saline and those receiving 0.4 x LD_{50} sarin became significantly ($p<0.05$) easier to remove from their cages when comparing FOB scores after the 10^{th} injection with those scores obtained as baseline (Table 1). The average "cage removal" score (mean ± S.E.M on a scored scale from 1 to 3) went from 1.50 ± 0.13 to 1.04 ± 0.04 and from 1.83 ± 0.2 to 1.0 ± 0.0 for the saline and 0.4 x LD_{50} sarin animals, respectively. The guinea pigs receiving saline and 0.4 x LD_{50} sarin also became significantly ($p<0.05$) easier to handle over the same time period. The average "handling" score (mean±S.E.M on a scored scale from 1 to 4) went from 2.54 ± 0.15 to 2.04 ± 0.04 for the animals receiving saline and from 2.50 ± 0.12 to 1.96 ± 0.04 for the animals receiving 0.4 x LD_{50} sarin. The guinea pigs receiving saline also developed significantly ($p<0.05$) decreased approach (2.04 ± 0.19 to 1.29 ± 0.14 on a scored scale from 1 to 6) and touch (2.21 ± 0.2 to 1.46 ± 0.2 on a scored scale from 1 to 6) responses

over the same period. In contrast, the animals receiving 0.4 x LD_{50} sarin failed to show any significant decreases in their approach and touch response scores when comparing their baselines with their scores after the 10^{th} sarin injection. The 0.5 x LD_{50} sarin animals failed to show significant changes in removal from cage, touch response and approach response, and they did not adjust to handling. No significant changes in FOB scoring was observed for any of the other measurements (lacrimation, salivation, fur appearance, latency to move, # grooms, # rears, arousal, gait, fecal boluses, urine spots, click response, righting reflex or drop reflex) in any of the three groups (Table 1).

A two-way repeated measures ANOVA of gait angles revealed significant main effects for treatment group ($F_{(2, 52)} = 16.05$, $p < 0.001$) and days ($F_{(7, 364)} = 2.04$, $p = 0.05$); the treatment group x days interaction was not significant. These data are displayed in Figure 3. The 0.5 x LD_{50} sarin group had significantly greater gait angles than did the saline controls or the 0.4 x LD_{50} sarin group; there was no difference between the gait angles of the animals receiving saline and those receiving 0.4 x LD_{50} sarin. In general, it was observed that the guinea pigs injected with 0.5 x LD_{50} sarin tended to keep their hindfeet parallel to each other and hop rather than alternate steps as did the saline control and 0.4 x LD_{50} sarin guinea pigs. This is illustrated by measuring the angle between consecutive footprints (Figure 4). Additionally, the drop reflex was impaired in 2/9 guinea pigs treated with 0.5 x LD_{50} sarin; this was never observed in the saline controls or animals receiving 0.4 x LD_{50} sarin. It was also observed that the animals receiving 0.5 x LD_{50} sarin showed obvious muscle tremors upon attempts to move. In contrast, muscle tremors were never observed in the animals receiving the 0.4 x LD_{50} dose of sarin. Hindlimb foot splay measurements were nearly identical for the saline, 0.4 and 0.5 x LD_{50} sarin guinea pigs (Figure 5) throughout the 10 days of injections and during recovery.

EEG spectral analysis

The total EEG power recorded over the baseline period for each day of exposure, as well as on the 6^{th} day of recovery, was nearly identical for the animals receiving saline, 0.4 x LD_{50} sarin or 0.5 x LD_{50} sarin (Figure 6A). For the most part, on any given day of injection or recovery the total power would generally increase, when compared with the baseline power, as the periods moved further away from the injection time (i.e., P5>P4>P3>P2>P1). The average total powers for the P5 period (30-36 minutes after saline or sarin injection) on the 10th day of injections were 8510.97, 4759.43, and 2058.33 for the saline, 0.4 x LD_{50} and 0.5 x LD_{50} sarin animals, respectively. These values amount to 159, 93 and 41% of the power values obtained from the same individual groups of animals during the same time period on the first day of injections. The total powers, during the P5 period, on the 6^{th} day of recovery for the animals receiving saline, 0.4 and 0.5 x LD_{50} sarin were 8959.22, 6714.52 and 2965.58, respectively. These values amount to 167, 131 and 59% of the power values obtained from the same individual groups of animals during the same time period on the first day of injections. There were noticeable differences in total spectral EEG power for the saline, 0.4 x LD_{50} sarin and 0.5 x LD_{50} sarin groups. When the powers were broken down into frequency bandwidths the most striking changes were in the delta (Figure 6B) and theta (Figure 6C) bands, with some changes in the alpha (Figure 6D) and beta I (Figure 6E) bands. There was a significant increase ($p<0.05$), when compared to the same period on day 1, in high-frequency beta II activity (Figure 6F) during the baseline period on day 10 and after 6 days of recovery in both groups (0.4 and 0.5 x LD_{50} sarin) receiving the sarin injections. However, the significant increase in beta II at 6 days recovery was also present in the animals that had received saline.

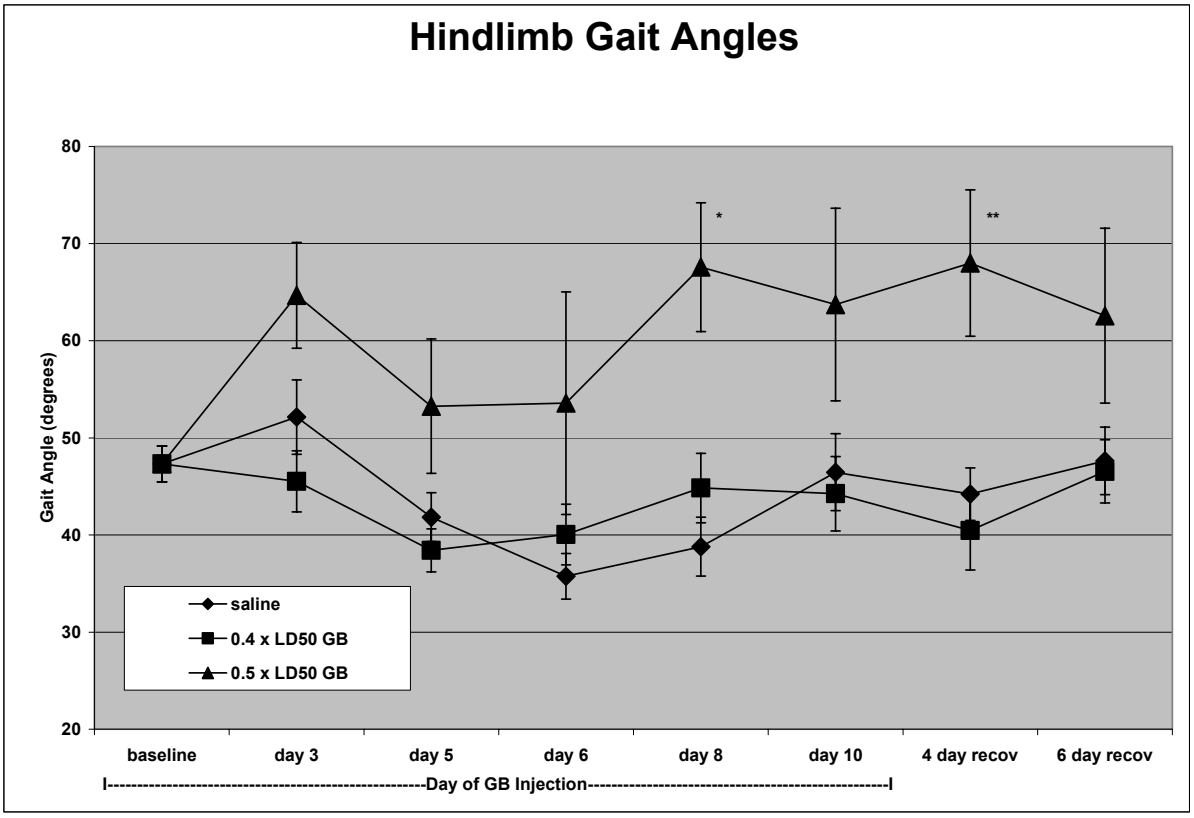


Figure 3. Gait angle vs. day of sarin injection. This figure shows the changes in the guinea pig's angle of gait vs. the day of injections for saline and the different concentrations of sarin (0.4 x LD_{50} and 0.5 x LD_{50}). The gait angle is measured as described in the methods section. Values are expressed as means ± S.E.M. n = 24 for saline group, n = 24 for 0.4 x LD_{50} sarin group, n = 7 for 0.5 x LD_{50} sarin group. (There were 9 guinea pigs total, but 2 guinea pigs died before the 8^{th} day of sarin injections and thus were not able to be included in statistical analysis using a two-way repeated measures ANOVA.) A repeated measures ANOVA showed that overall (main effect) the 0.5 x LD_{50} sarin group had significantly greater gait angle than the saline controls or the 0.4 x LD_{50} sarin group, which did not differ from one another. The average angle between hindlimb steps is exaggerated in the 0.5 x LD_{50} sarin group because they tended to hop rather than walk.

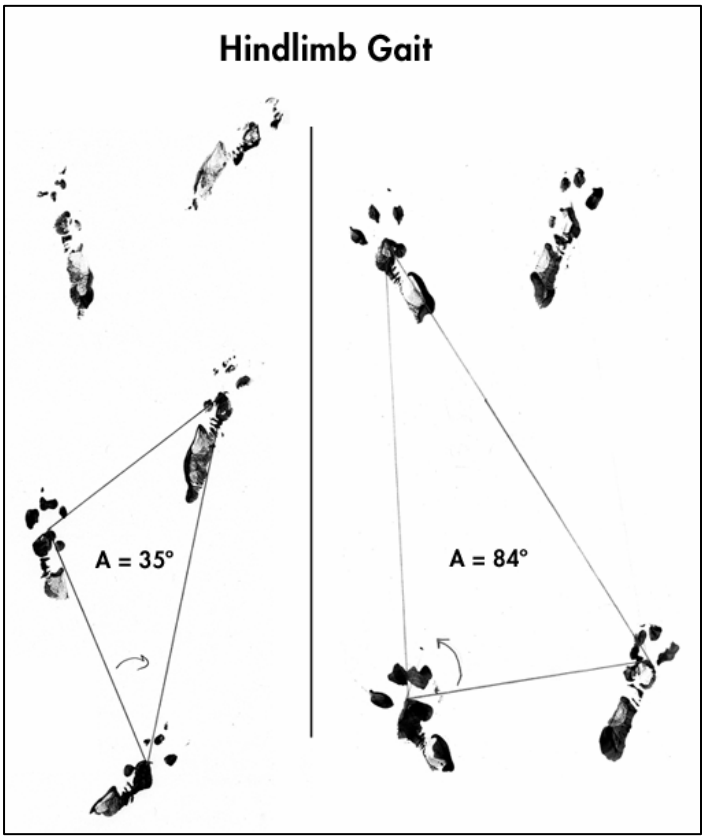


Figure 4. Hindlimb Gait. These figures are representative samples of guinea pig hindlimb gaits. The footprints on the left are from a saline control guinea pig after the 6th day of injections. The footprints on the right are from a guinea pig that had received 10 injections of 0.5 x LD_{50} sarin. The animals were required to walk in a relatively straight line for a minimum of 3 successive steps. Lines were drawn from a similar point on each foot, and the angle that is formed between the furthest foot back, the opposite foot, and the next step of the first foot was measured with a protractor.

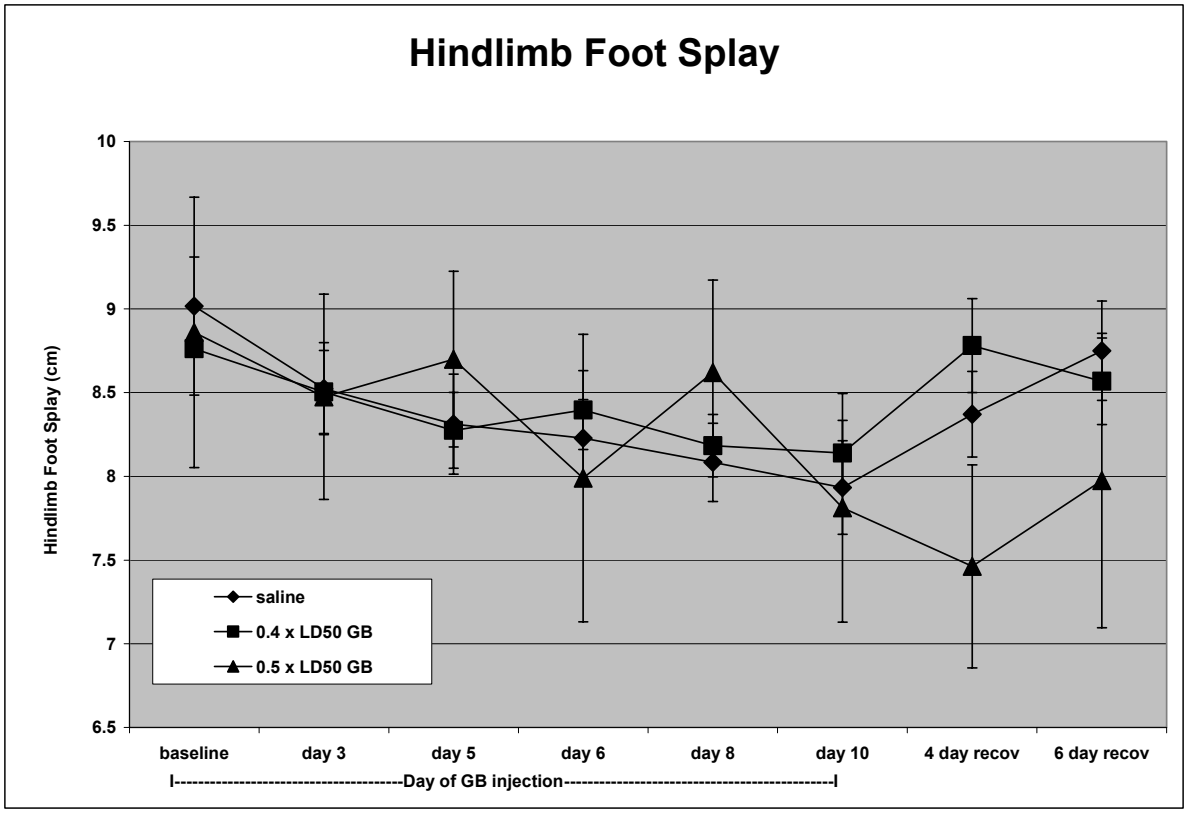
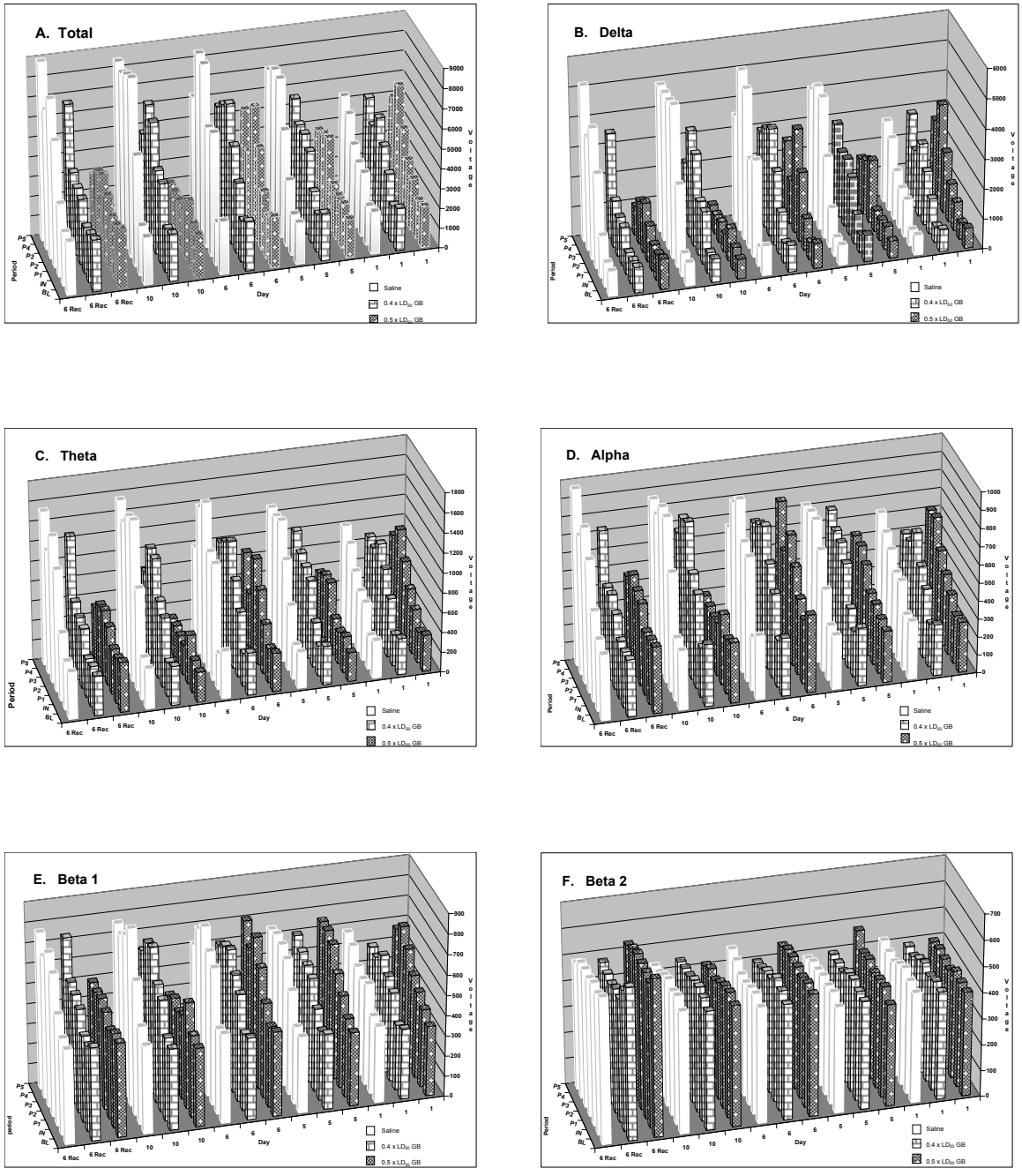


Figure 5. Hindlimb foot splay vs. day of sarin injection. This figure shows the hindlimb foot splay (in cm) vs. the day of sarin injections for the saline, 0.4 and 0.5 x LD_{50} sarin groups. The animal's feet were painted with tempura paint, and the animal was dropped from approximately 30 cm onto a piece of paper. The distance between the footpads under the middle toe on each foot was measured. Each drop was performed in duplicate and averaged (n = 24 for saline group, n = 24 for 0.4 x LD_{50} sarin group, and n = 7 for 0.5 x LD_{50} sarin group). The values are expressed as means ± S.E.M. There are no statistical differences between the hindlimb splay of the guinea pigs injected with saline vs. those injected with either 0.4 or 0.5 x LD_{50} sarin.

Figure 6.



Figures 6A-F. EEG total and band powers. The EEG power spectra are graphed as a function of day of injections with time period reflected on the z-axis. Each bar reflects the mean for that particular day and time period taken over a 120-second time snippet within the time period. Each 120-second time snippet was taken as close to the beginning of the time period as possible (with the exception being the BL time period where the snippet was taken in the 120 seconds prior to the injection being given). The time periods were as follows: BL= the baseline period before injections; IN = 0-6 minutes following injection; P1= 6-12 minutes following injection; P2 = 12-18 minutes following injection; P3 = 18-24 minutes following injection; P4 = 24-30 minutes following injection; and P5 = 30-36 minutes following injection.

The total EEG cortical power consisted of bandwidth powers over 5 different frequency ranges as follows: Delta = 1-3.5 Hz; Theta = 4-7.5 Hz; Alpha = 8-12.5 Hz; Beta I = 13-20.5 Hz; and Beta II = 21-31.5 Hz.

For each of the EEG spectral graphs the white bars, bricked bars, and dotted bars are the average powers from animals receiving saline, 0.4 x LD_{50} sarin and 0.5 x LD_{50} sarin, respectively. On days 1, 5, 6, and 10 n = 30 for the animals receiving saline or 0.4 x LD_{50} sarin. On the 6^{th} day of recovery n = 15 for both the saline and 0.4 x LD_{50} sarin groups. In the 0.5 x LD_{50} sarin group n = 16 for day 1, n = 12 for days 5 and 6, n = 11 for day 10 and n = 7 for 6 day recovery.

Neurochemistry

Ten to twelve days after the final sarin (0.4 x LD_{50} sarin) injection, while there was a measurable difference between the mean (± S.E.M.) values of choline in the sarin-treated animals, when compared to the saline-treated animals, the difference failed to reach significance at any time (Figure 7). The trend towards a reduction in choline from the start of fraction collection in the saline-treated animals could be attributed to a gradual decrease in remaining endogenous AChE activity in the brain due to the inclusion of neostigmine in the perfusion buffer. The neostigmine (a carbamate AChE inhibitor) prevents the breakdown of ACh into choline. In the sarin-treated animals AChE activity was already inhibited because of the actions of the nerve agent. Therefore, there was a resulting decrease in choline formed as a byproduct of ACh breakdown.

Baseline levels of ACh (calculated as the average of the last 2 fractions before atropine sulfate injection) were slightly higher, although not significantly, in the sarin-treated animals than in saline-treated animals (data not shown). Analysis of the ACh data showed no significant group effect, nor group x time interaction. There was, however, a significant effect of time, with ACh values at the 45-, 60-, 75- and 90-min time points relative to the 15-min time point (Figure 8). This demonstrates the stimulating effects of atropine on ACh output. In addition, simple main effect tests showed that the sarin-treated group contributed the most to this increase; there were no significant differences in ACh levels of the saline-treated group over the 8 time points, while for the sarin-treated group ACh was significantly elevated above their 15 min levels at the 60- and 90-min samples ($p<0.05$) and the 75-min sample elevation just missed the conventional level of significance ($p<0.07$).

Pathology

No evidence of brain or heart pathology was found in any guinea pig that survived all 10 sarin (0.3, 0.4 or 0.5 x LD_{50}) injections. Of the animals that died during experimentation no brain or heart tissue was submitted for postmortem examination, because those animals died overnight and were not found until the following morning. It was verified in each case that the tip of the brain microdialysis probe was within the caudate nucleus.

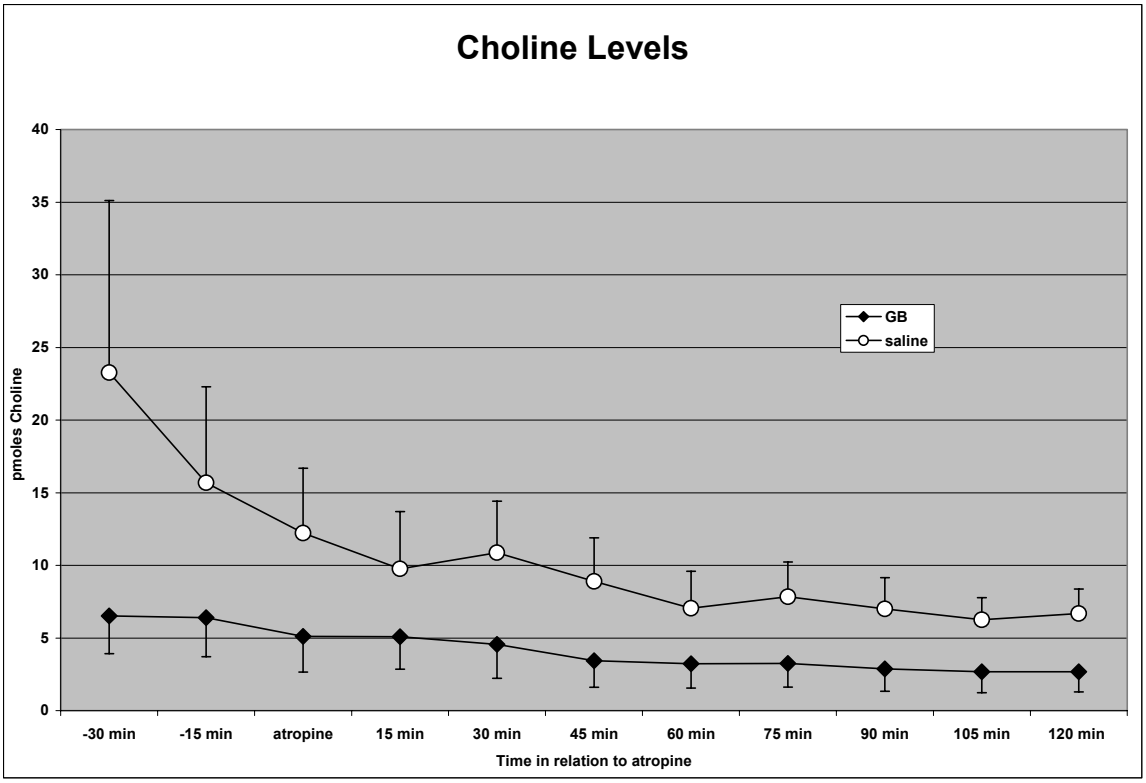


Figure 7. Striatal choline levels in sarin-treated vs. saline-treated guinea pigs. Saline- (○) and 0.4 x LD_{50} sarin-(♦)treated guinea pigs were allowed to recover for 10-12 days before undergoing microdialysis collection from the caudate nucleus. The amount (pmoles) of choline (mean+S.E.M.), contained in one 15-minute fraction (3 µl/min) of dialysate, is graphed against the time of the fraction in relation to the injection of atropine sulfate (5 mg/kg, i.p.). While there is a measurable difference between the mean (± S.E.M.) values of choline in the sarin-treated animals as compared with the saline-treated animals, the difference fails to reach significance at any time. The trend towards a reduction in choline from the start of fraction collection in the saline-treated animals can be attributed to a gradual decrease in remaining endogenous AChE activity in the brain due to the inclusion of neostigmine in the perfusion buffer. The neostigmine prevents the breakdown of ACh into choline. In the sarin-treated animals AChE activity is already inhibited because of the actions of the nerve agent. Therefore, there is a resulting decrease in choline formed as a byproduct of ACh breakdown.

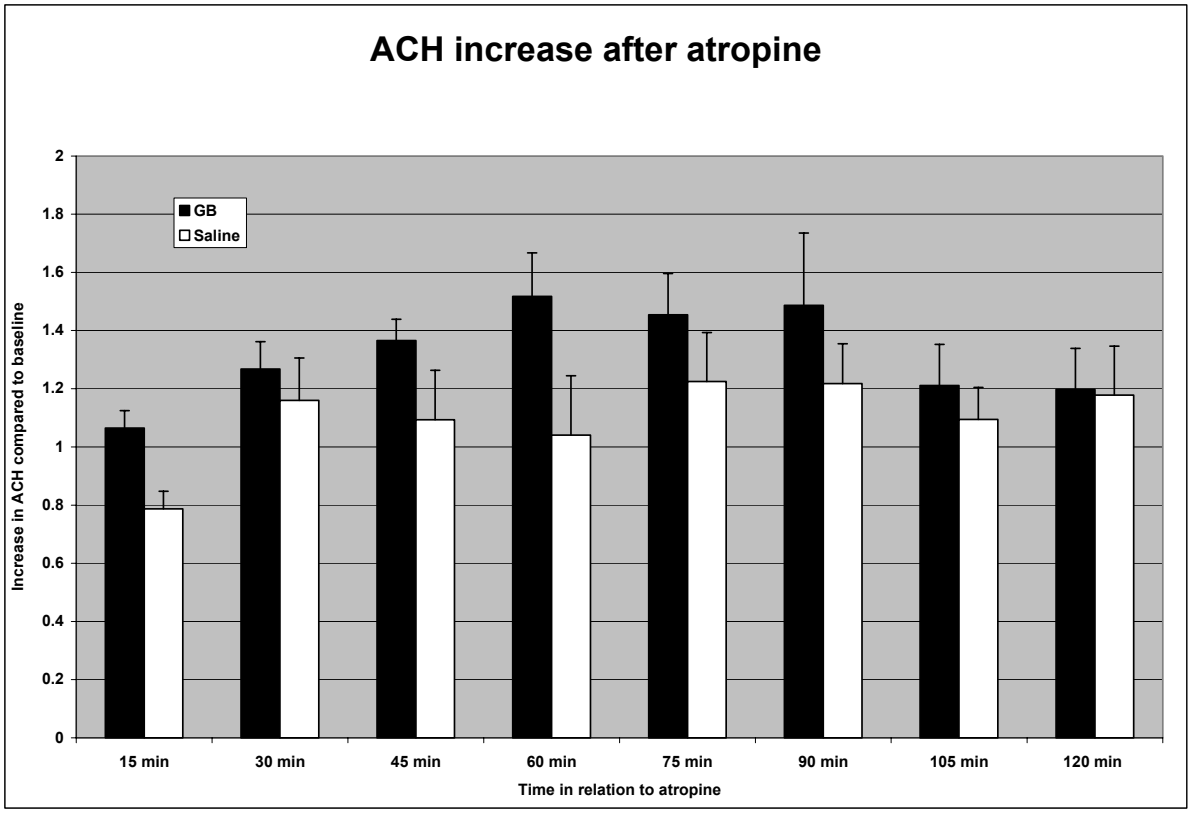


Figure 8. ACh in sarin-treated vs. saline-treated guinea pigs. The increase in measurable ACh (mean ±S.E.M.) as compared with baseline values before atropine sulfate injection has been graphed for the 0.4 x LD_{50} sarin-(dark bars) and saline-(light bars) treated guinea pigs vs. time after atropine sulfate (5 mg/kg, i.p.) injection. The data have been normalized to take into account the differences between baseline ACh values between the 2 groups. Therefore, a value of 1 on the Y-axis is equal to a value of ACh (pmols) that is 100% of the baseline measurements prior to atropine injection. Analysis of the ACh data showed no significant group effect, nor group x time interaction. There was a significant increase in ACh levels in both groups as a function of time; ACh levels at 45-, 60-, 75- and 90-min (#) were elevated relative to the 15-min sample. Simple main effects showed that the sarin-treated group contributed most to this effect; the samples of the 60- and 90-min time-points (*) being significantly elevated compared to the 15-min sample. Only at the 15-min time point (♣) was there a significant increase in ACh levels of the sarin-treated group relative to the saline controls.

DISCUSSION

One of the objectives of the current set of experiments was to establish a dose of sarin to be utilized in a model for further study of the effects of low-dose repeated exposure to sarin in the guinea pig model. A primary requirement of the model was to obtain a dose of sarin that could be given over a 2-week period of exposures without causing easily identifiable cholinergic signs such as tremors, EEG epileptiform seizures or death. Although no guinea pigs in the 0.5 x LD_{50} sarin group showed signs of epileptiform seizures, 2 of 13 (15%) animals died before all 10 sarin injections were given. This dose also caused a rapid decrease in RBC AChE levels to approximately 10% of baseline values by the 2nd day of sarin injections. Additionally, there were noticeable signs of sarin intoxication (chewing, hyperactivity, muscle tremor), alterations in angle of gait (Figure 3) and impaired drop reflexes in some of the guinea pigs receiving 0.5 x LD_{50} sarin. Youssef and Santi (1997) showed that multiple low-dose injections of either acrylamide or methanol, both known neurotoxicants, resulted in changes in the angle of gait similar to those observed in the study presented here. Moser (1995) used similar neurobehavioral screening batteries after a single acute dose of 7 different AChE inhibitors to conclude that altered gait could be considered a "cardinal sign of toxicity for cholinesterase inhibitors." Because there was observational evidence (e.g., observed tremors when attempting to move) that gait abnormalities developed after the first 0.5 x LD_{50} sarin injection, this dose was most likely to be indicative of acute nerve agent poisoning. However, the gait problems identified in the animals receiving 0.5 x LD_{50} sarin were accompanied by significant reductions in body weight gains over the same periods. Since our experiments did not include a group solely to look at the effect of weight loss on gait we cannot completely rule out its effect. In contrast to the signs of acute nerve agent toxicity witnessed in guinea pigs that received 0.5 x LD_{50} sarin, the animals receiving 0.4 x LD_{50} sarin showed no signs of acute toxicity. This finding confirmed the report of Atchison et al. (2004) that in guinea pigs dosed once daily for 2 weeks (using the same exposure protocol as the current study) the MTD was 0.4 x LD_{50} for sarin. The fact that we found no pathological evidence in any of the animals that received all 10 sarin (0.3, 0.4 or 0.5 x LD_{50}) injections (cumulative doses of 3, 4 and 5 x LD_{50}, respectively) indirectly supports prior studies showing that nerve agent-induced brain pathology is due to seizures (McDonough et al., 1989, 1995; Shih et al., 2003) and not total dose of nerve agent.

By the 2nd day of sarin injections, RBC AChE levels in animals receiving the 0.5 x LD_{50} dose had dropped significantly ($p<0.001$) lower (14% of baseline values) than the RBC AChE levels in animals receiving 0.4 x LD_{50} sarin (Figure 2). The parallels between the onset of signs following the second 0.5 x LD_{50} sarin injection and the rapid reduction of the RBC AChE levels to approximately 14% of control values after the 2nd injection are consistent with the results of Grob and his associates. They found that the onset of signs upon human exposure to DFP (Grob et al., 1947) or sarin (Grob and Harvey, 1958) was correlated with rapid decreases of RBC AChE levels to 30 and 22% of baseline values, respectively. However, when the OPs were administered at lower doses over several days, there was no correlation between the onset of signs and RBC AChE levels (Grob et al., 1947). This is consistent with our studies in which RBC AChE in the animals receiving 0.4 x LD_{50} sarin had dropped to 11% of baseline values by the 10th day of sarin injections. The animals receiving 0.4 x LD_{50} sarin showed no obvious signs of nerve agent intoxication despite RBC AChE levels being nearly identical to those of the RBC AChE levels in the animals receiving 0.5 x LD_{50} sarin after the 10th sarin injection. While the RBC AChE levels in the guinea pigs receiving 0.5 x LD_{50} sarin dropped to approximately 10%

of baseline values after the 5th day of injections, these levels were maintained between 9 and 12% of baseline values despite 5 more injections of 0.5 x LD_{50} sarin. Additionally, the RBC AChE levels in the guinea pigs receiving 0.4 x LD_{50} sarin never fell below 10% of baseline values. It is commonly accepted that the only way that RBC AChE can be replaced once it is irreversibly inhibited by nerve agents is by *de novo* synthesis (Harris et al., 1971) of new RBCs, which is thought to occur at the rate of 1-2% per day (Grob and Harvey, 1958). Therefore, we must rule out the possibility that daily replenishment alone of RBC AChE is accounting for the failure of RBC AChE levels in our animals to fall below 10% of baseline values. The failure of RBC AChE to fall below 10% of baseline levels is most likely a combination of *de novo* RBC AChE synthesis (Grob and Harvey, 1958; Harris et al., 1971) and an increasing rate of spontaneous reactivation of RBC AChE associated with repeated exposures to nerve agents (Lanks et al., 1977).

The increase in EEG spectral power that the saline animals showed from the time of injection (BL) to 30-36 minutes after injection (P5) was more pronounced at the lower frequency bandwidths. It is most likely that the increase in total (specifically lower frequency) band powers from the time of injection to 36 minutes after injections was a result of the animals falling asleep. Unlike the animals that received saline, animals that received 0.4 x LD_{50} sarin or 0.5 x LD_{50} sarin for 10 exposures showed no increases in power over the time periods during the later sarin exposures. The differences in total and lower frequency band powers between the 0.5 x LD_{50} sarin animals and the saline animals lasted into the 6th day of recovery. While our findings of significant changes in high frequency beta II spectra support purported changes in beta II powers reported by Burchfiel et al. (1976), we also saw similar changes in animals that had received saline. These data suggest that repeated low-dose sarin exposures may disrupt normal cortical EEG sleeping patterns. While these disruptions were able to return to relative normalcy 6 days after the last injection in the animals receiving 0.4 x LD_{50} sarin, this change persisted in the 0.5 x LD_{50} sarin group for at least 6 days after the last injection.

There were no statistical differences found for many of the FOB scoring criteria (lacrimation, salivation, fur appearance, latency to move, numbers of grooms, numbers of rears, arousal, gait, fecal boluses, urine spots, click response, righting reflex or drop reflex) for animals receiving saline, 0.4 or 0.5 x LD_{50} sarin (Table 1). However, the 0.4 and 0.5 x LD_{50} sarin-treated animals showed decreased ability to habituate to certain aspects of the neurobehavioral testing, unlike their saline control counterparts. One important point is that the guinea pigs used for the experiments were handled very minimally prior to the first day of injections. When comparing the FOB scores obtained after the 10th day of saline injections with those obtained at baseline it is most likely that the significant changes observed in removal from cage and handling were due to the guinea pigs becoming acclimated to being handled by the technician scoring the FOB. It is also most likely that the saline-treated guinea pigs became less reactive to an approaching pencil (approach response) and to a touch on the rump (touch response) because of this habituation. In contrast, the animals receiving 0.4 or 0.5 x LD_{50} sarin failed to acclimate to some aspects of the FOB testing (approach and touch responses for the 0.4 x LD_{50} sarin dose, and handling, cage removal and approach and touch responses for the 0.5 x LD_{50} sarin dose). It is worthy of noting that the FOB scores after the 10th day of sarin injections, for both the 0.4 and 0.5 x LD_{50} sarin doses, were not "worse" than they were at baseline measurements. In short, the animals receiving sarin showed subtle neurobehavioral changes in that they demonstrated less ability to adapt to the FOB than did the guinea pigs receiving saline. It was interesting to note that in our previous study (Shih et al., 1990) in which rats were injected (s.c.) with 0.4 x LD_{50} of soman

once a day and 3 times (Mondays, Wednesdays and Fridays) a week for up to 6 weeks, the animals became hyper-reactive to normal handling procedures and demonstrated exaggerated startle responses to air puffs.

Thus, guinea pigs receiving 0.4 x LD_{50} sarin for 2 weeks of daily repeated exposure (Mondays - Fridays) were virtually indistinguishable in gross behavior and body weight changes from those that received saline over the same time period. In spite of these observations, animals that received 0.4 x LD_{50} sarin showed a decrease of their RBC AChE to approximately 10% of baseline levels by day 10. The 0.4 x LD_{50} sarin dose inhibited RBC AChE levels to approximately that of the 0.5 x LD_{50} group without the subsequent signs of acute sarin exposure (hyperactivity, chewing, gait impairment, impaired reflexes, etc.). However, the FOB revealed that they showed very subtle neurobehavioral changes. The understanding of whether these subtle neurobehavioral changes can last for extended post-exposure periods and whether these neurobehavioral changes can progress in severity may be important for investigating the developing health hazards of long-term exposure to CWNAs. Because of these reasons, the 0.4 x LD_{50} dose of sarin is more suitable for utilization as a model for sub-symptomatic non-acute repeated sarin exposure in the guinea pig. Therefore, we utilized this model of repeated non-acute sarin dosing in conjunction with *in vivo* microdialysis to assess NT changes, such as ACh and choline, in brain over the post-injection period.

By the basic nature of an OP nerve agent's ability to inhibit AChE activity both peripherally and centrally one would suspect that there would be a measurable elevation of ACh within the brain, even 10-12 days after the final sarin exposure (based on RBC AChE recovery data shown in Figure 2). However, in our studies, there was no statistical difference between the levels of striatal ACh from animals that had received 0.4 x LD_{50} sarin injections vs. those that had received saline injections. Two possible explanations for this come to mind: 1) the 10-12 days between the last sarin injection and the taking of the dialysate samples was sufficient for striatal AChE levels to return to normal, or 2) the prolonged inhibition of extracellular AChE led to a prolonged increase in extracellular ACh. The prolonged availability of ACh in the synaptic cleft results in feedback inhibition on muscarinic, pre-synaptic autoreceptors and, thus, a decrease in further ACh release (Russell et al., 1985). Should the latter of these hypotheses be correct then we would expect that a sudden release of the feedback inhibition would result in a rapid release of ACh into the cleft. Indeed this may be the case. Atropine works as a competitive ACh antagonist at both pre- and post-synaptic receptors and, therefore, is able to terminate presynaptically the controls of feedback inhibition. Within 15 minutes after the atropine injection there was a substantial (approximately 1.5 times) increase in ACh in the striatal dialysate as compared with the increase in ACh dialysate from saline controls. However, this effect was only transient, since the saline-treated animals also showed increased ACh after atropine challenge. The results of the measurements of choline within the dialysate also appear to support the latter hypothesis. The observable trend of a reduction in measurable choline in the animals receiving sarin compared with those receiving saline is most likely due to the decrease in AChE in the cleft of animals treated with sarin. This would lead to a decrease in ACh breakdown and, therefore, a reduction in measurable choline. Indeed the initial high choline measurements, evident in the animals that had received saline, begin to decrease to approximately the same choline levels seen in the animals that had received sarin, as more neostigmine was infused with the dialysate buffer. Neostigmine was thus acting in the same manner as the nerve agent; decreasing the breakdown of ACh results in a decrease of its breakdown products, the major component being choline. These trends of neurochemical results

at 10-12 days after 2 weeks of 0.4 x LD_{50} sarin injections led us to speculate that the normal brain NT and receptor homeostasis might have been disrupted, at least in the striatum, during or soon after repeated subacute sarin exposures. It is, therefore, speculated that these alterations in brain chemistry may be the pharmacological basis for the neurobehavioral and EEG changes observed in the present study.

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ABBREVIATIONS

ACh = acetylcholine
AChE = acetylcholinesterase
CWNA = chemical warfare nerve agent
DFP = diisopropylfluorophosphate
DOPAC = 3,4-dihydroxyphenylacetic acid
EEG = electroencephalographic activity
FOB = functional observational battery
HPLC = high pressure liquid chromatograph
HVA = homovanillic acid
i.m. = intramuscular
i.p. = intraperitoneal
LC_{50} = median lethal concentration
LD_{50} = median lethal dose; MTD = maximum tolerated dose
NT = neurotransmitter
OP = organophosphorus compound
RBC = red blood cell
s.c. = subcutaneous

APPENDIX

FUNCTIONAL OBSERVATIONAL BATTERY SCORE SHEET

Date_____ Guinea pig #_____ Weight_____
Scoring code_____ Time started_____ Scorer_____

Home Cage Assessment

Agitated	___yes	_____no
Chewing	___yes	_____no
Facial dysmorphia	___yes	_____no
Tremors	___yes	_____no
Vocalizations	___yes	_____no

Animal handling

Ease of removal from cage: (R) (choose one)

1. Easy; little or no vocalization, without resistance
2. Moderately Easy; animal jumpy, initial movement followed by settling, with or without vocalizations
3. Difficult; runs around cage, is hard to grab, with and without vocalizations

Ease of Handling Guinea Pig in hand: (R) (choose one)

1. Easy, but lethargic
2. Easy, but alert, limbs may be pulled against body
3. Moderately easy; vocalizations, little or no squirming
4. Difficult, squirming, twisting, attempting to bite, with or without vocalizations

Lacrimation: (R)
1. none
2. slight
3. severe
4. rough

Salivation: (R)
1. none
2. slight
3. severe

Fur Appearance: (R) (choose one for each)
1. normal
2. slightly soiled/disheveled
3. very soiled/crusty

Open Field Checklist (2 minutes)

Latency to first movement (sec)_____ Total # of rears (C) _____
Total # of grooming episodes (C)_____

Arousal: (R) (choose one)

1. Very low (little or absent)
2. Low (some head or body movement)
3. Somewhat low (some exploratory movements with period of immobility)
4. Normal (alert, exploratory movements)
5. Somewhat high (slight excitement, sudden darting or freezing)
6. Very high (hyper-alert, excited, sudden bouts of running or body movements)

Gait description: (D) (choose one)

1. No movement
2. Normal
3. Impairment
    a. Uncoordinated movement (ataxia)
    b. Walking on toes
    c. Splayed hind limbs
    d. Exaggerated hind limb flexion
    e. Staggered gait
    f. Dragging hind limbs
    g. Unable to walk

Total # of fecal boluses (C)_____ Total # of urine spots (C)_____

Reflexes

Click Response: (R) (choose one)

1. no reaction
2. slight reaction, ear flick or some evidence that sound was heard
3. more energetic response than (2); may include vocalization
4. jumps, seems startled
5. freezes, actual muscle contraction
6. bizarre reaction: bites, attacks

Approach response: (R) (choose one)

1. no reaction
2. slow approach, sniffing or turning away
3. more energetic response than (2), possible vocalizations
4. jumps, makes efforts to avoid object
5. freezes, actual muscle contraction
6. bizarre reaction: bites, attacks

Touch Response: (R) (choose one)

1. no reaction
2. slowly turns, walks away
3. more energetic response than (2), possible vocalizations
4. jumps, makes efforts to avoid object
5. freezes, actual muscle contraction
6. bizarre reaction: bites, attacks

Gait scoring: (C) (1 trial)

Stride length (cm)_____
Stride width (cm)_____
Angle_____

Foot Splay Measurements (2 trials) trial 1(cm)_____ trial 2 (cm)_____

Righting Reflex: (R)

1. normal (immediately rights itself)
2. slightly impaired (>1 sec)
3. impaired (>2 sec)
4. totally impaired (remains on back)

Drop Reflex: (R)

1. normal
2. slightly uncoordinated
3. lands on side
4. lands on back

COMMENTS: